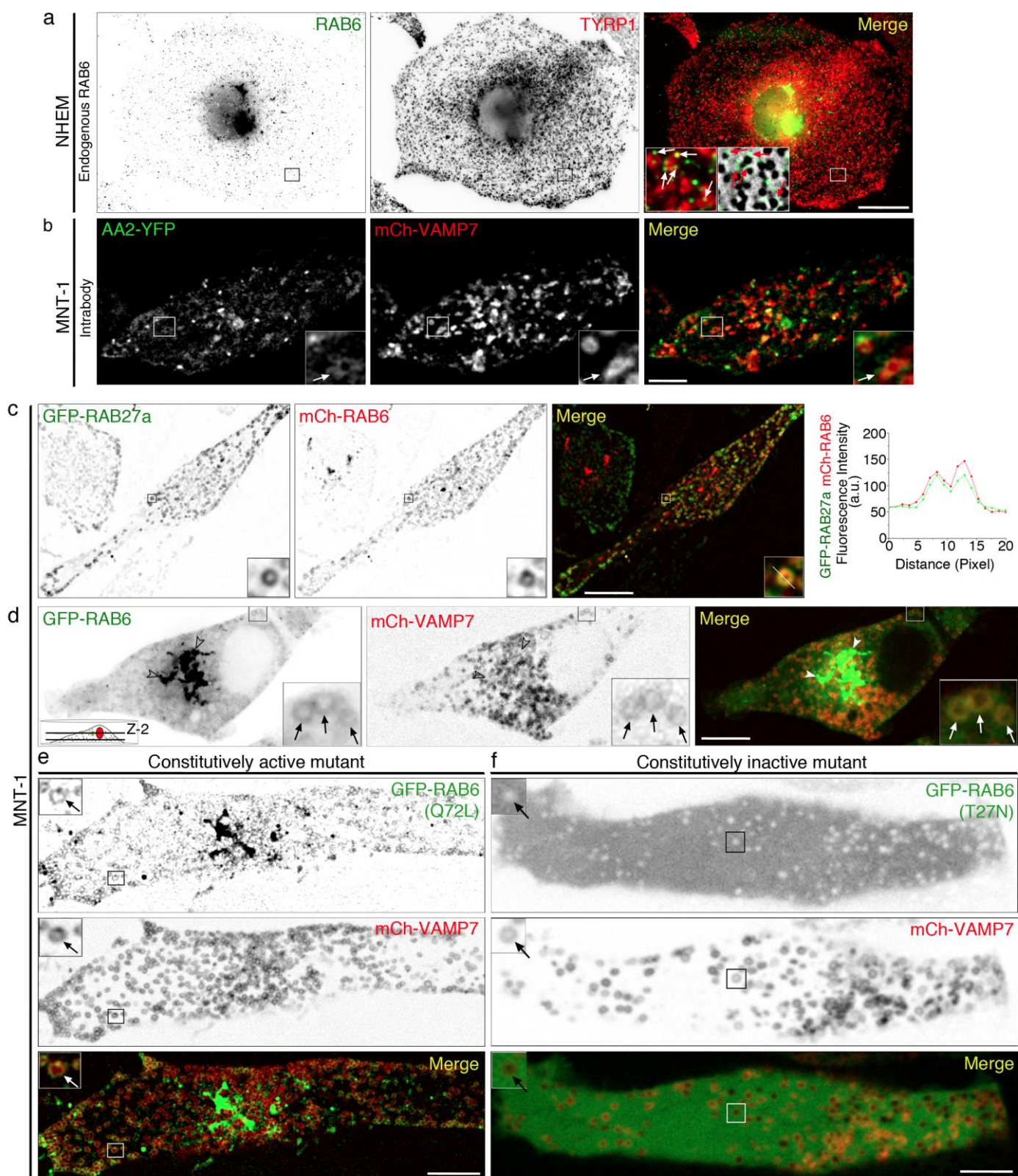


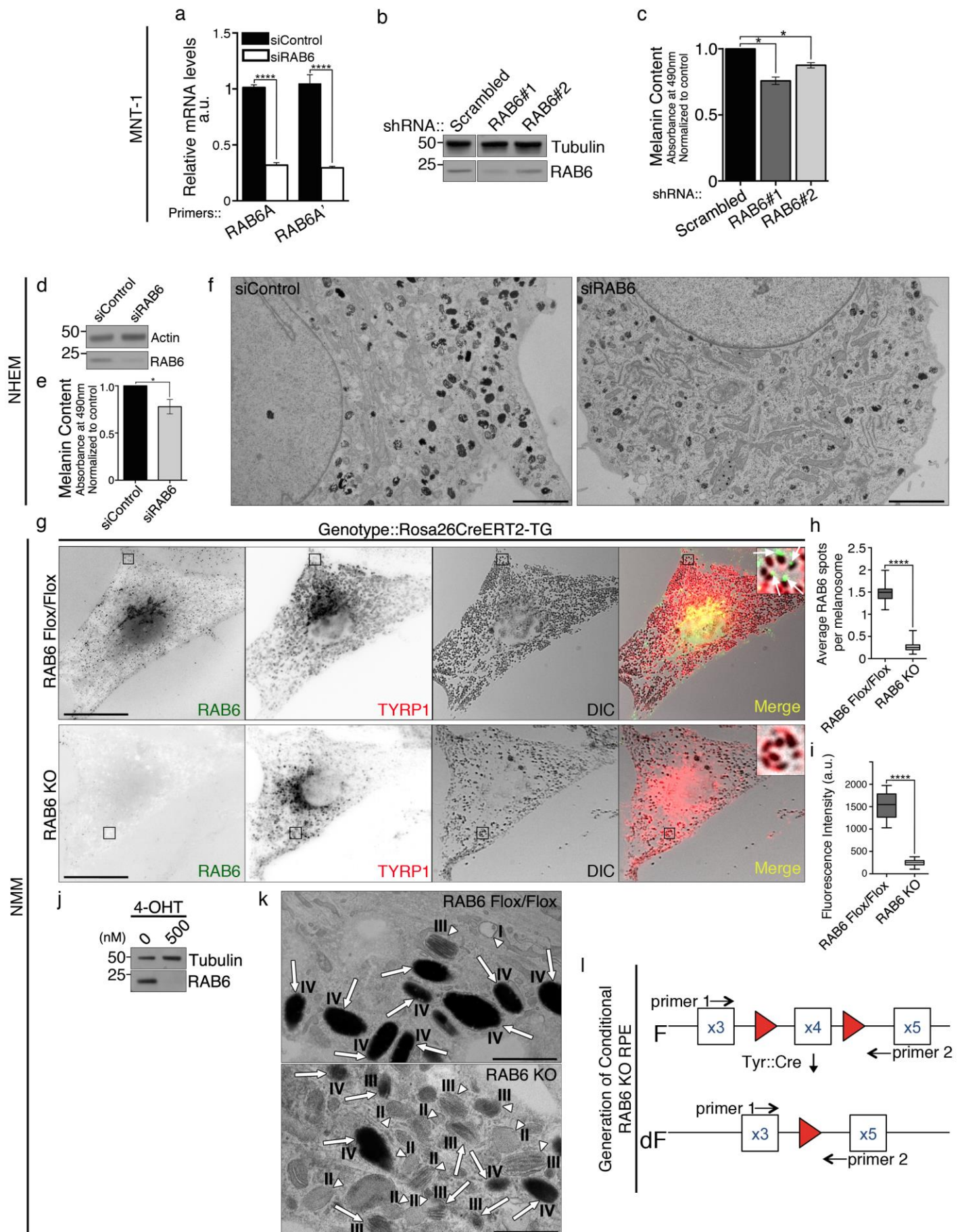
Supplementary Figure 1



Supplementary Figure 1: RAB6 associates to melanosomes in a GTP-dependent manner

(a) IFM on Normal Human Epidermal Melanocytes (NHEM) stained for endogenous RAB6 and TYRP1 showing RAB6 association with TYRP1- and melanin-positive melanosomes (white and red arrows, respectively, 5X area). (b) Live imaging frame of MNT-1 cells co-expressing AA2-YFP (RAB6:GTP intrabody) and mCh-VAMP7. AA2-YFP localized as punctate or donut-like structure (arrow) co-distributed with mCh-VAMP7 (3X area, see also **Supplementary Movie 1**). (c) Live imaging frame of MNT-1 cells expressing GFP-RAB27a and mCh-RAB6. Corresponding intensity profile of RAB6 across melanosome was measured along the white line (5X area). (d) Live imaging frame of MNT-1 cells co-expressing GFP-RAB6 and mCh-VAMP7 (imaging plane Z-2 of the same cell as in Figure 1d) showing RAB6 distribution to the Golgi area (arrowheads) and to VAMP7-positive melanosomes (arrows, 5X area). (e-f) Live imaging frame of MNT-1 cells expressing GFP-RAB6 mutants and mCh-VAMP7 that showed melanosome-associated activated GFP-RAB6 (Q72L mutant, arrows, e), or cytosolic inactive mutant (T72N, f). Scale bars: 10 μ m (a-f).

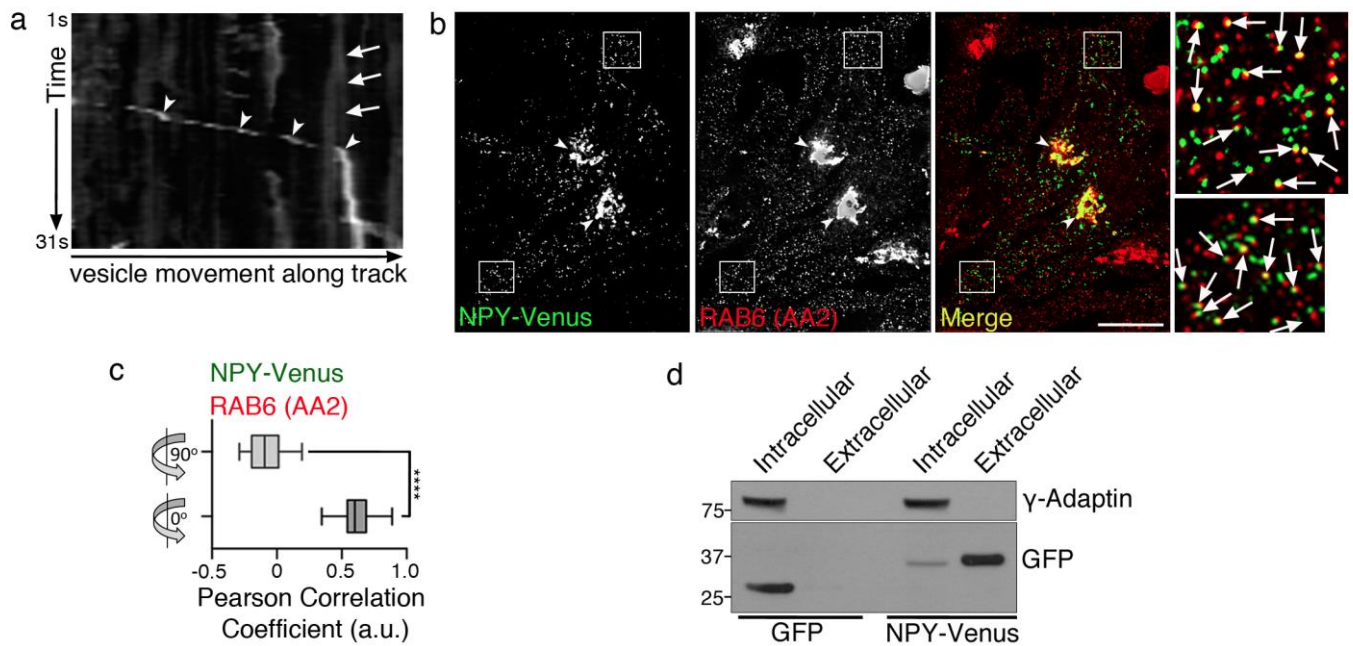
Supplementary Figure 2



Supplementary Figure 2: *In vitro* or *in vivo* RAB6 extinction impacts pigmentation

(a) Relative mRNAs expression levels of RAB6 isoforms (A & A') by real time PCR of RAB6 siRNAs treated MNT-1 cells. Data are normalized to 26s rRNA as control and presented as mean \pm SEM (3 independent experiments). (b) WB of control (scramble) or RAB6 shRNAs expressing MNT-1 stable cell lines; lysates were probed for RAB6 or Tubulin. (c) Melanin content estimation of cells treated as in b. Data are normalized to control and presented as means \pm SEM (3 independent experiments). (d) WB of control- or RAB6-siRNA depleted NHEM probed for RAB6 or Actin. (e) Melanin content estimation of siRNA-treated NHEM as in (d). Data are normalized to control and presented as means \pm SEM (3 independent experiments). (f) Representative electron micrograph by conventional EM on control- or RAB6-depleted NHEM cells. (g) IFM on NMM isolated from Rosa26CreERT2-TG mice treated (bottom, RAB6 KO) or not (top, RAB6 Flox/Flox) with 4-OHT (4-hydroxy tamoxifen) and stained for endogenous RAB6 and TYRP1. RAB6 localized in the vicinity of TYRP1- (red) and melanin-positive melanosomes (arrows), which disappeared upon 4-OHT treatment (5X area). (h-i) Quantification of the number of endogenous RAB6-positive structures associated to melanosomes (h) and overall RAB6 fluorescent intensity (i) in NMM treated as in (g) (90-100 melanosomes per cell, n=31-35 cells, 7 independent mice). (j) WB of ethanol- (untreated) or 4-OHT-treated NMM (as in (g)), probed for RAB6 or Tubulin as control. No change in RAB6 protein expression level was observed in *Rosa26CreERT2-TG^{-/-}*; *Rab6^{F/F}* mouse melanocytes (data not shown). (k) High magnification EM image from cells as in Figure 2e showing immature melanosomes (arrowheads) and pigmented melanosomes (arrows). Note the increase of immature organelles in RAB6 KO NMM. (l) Diagram (not to the scale) of the floxed (F) and defloxed (dF) Rab6 locus. LoxP sequences (red triangle) flank Rab6 exon 4 (x4) of the floxed allele. After appropriate crosses with Tyr::Cre transgenic mice, Rab6 exon 4 is deleted. The mice genomic DNA was amplified using primers 1 and 2 (arrow 1 and 2). The exons are represented as rectangles and the black line corresponds to the intronic sequence. Scale bars: 5 μ m (f), 20 μ m (g), 500 nm (k); Two-tailed, unpaired t tests (a, c, e, h, j); *, $P < 0.05$; **** $P < 0.0001$; M.W. in kDa.

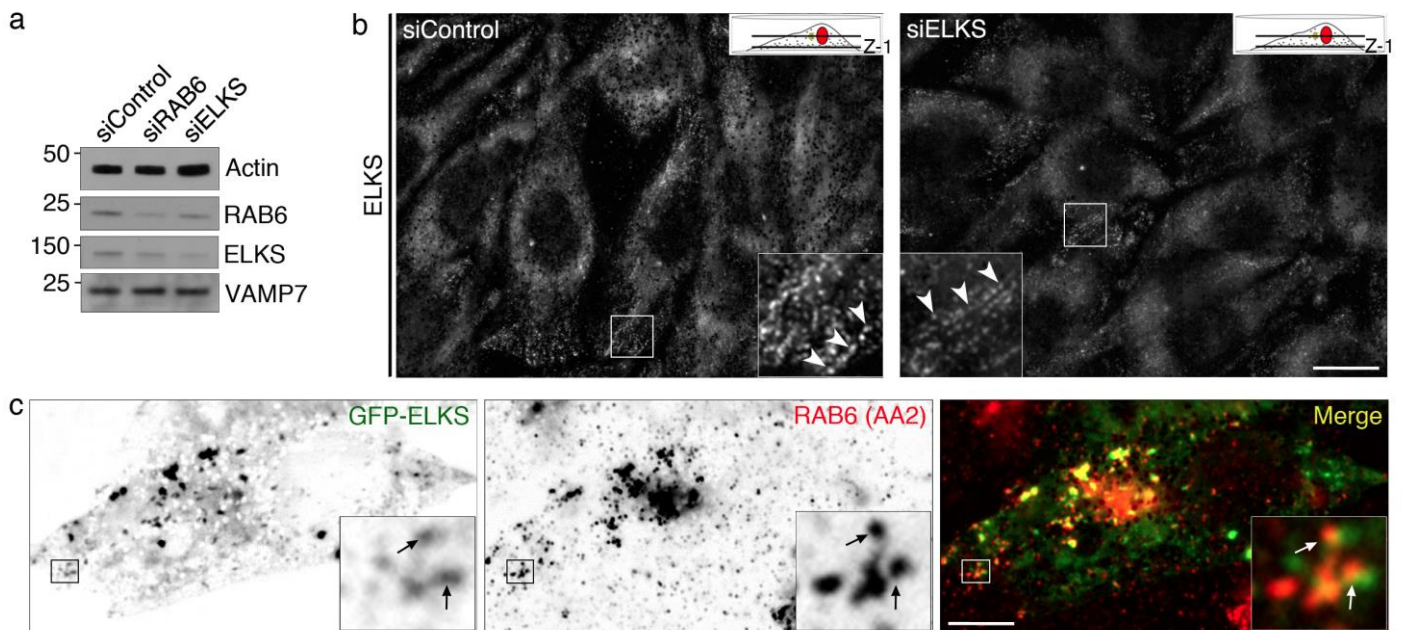
Supplementary Figure 3



Supplementary Figure 3: Exogenously-expressed NPY co-distribute with RAB6 in MNT-1 cells

(a) Representative kymograph (track from **Supplementary Movie 4**) of a motile GFP-RAB6-positive vesicle (arrowheads), immobilizing in the vicinity of a melanosome (arrows). (b) NPY-Venus expressing MNT-1 cells stained for endogenous RAB6:GTP (AA2) showed co-distribution on Golgi (arrowheads) and peripheral vesicles (arrows, 5X area). (c) Pearson's correlation coefficient of NPY-Venus and RAB6:GTP. Correlation was lost when one of the channels was rotated by 90° (n=34 cells, 4 independent experiments). (d) WB of GFP or NPY-Venus expressing MNT-1 cell lysates and supernatant recovered 24h post-transfection and probed for GFP or γ-adaptin (subunit of adaptor protein complex 1, as intracellular protein control). Scale bar: 10 μm; Two-tailed, unpaired t tests; **** P < 0.0001; M.W. in kDa.

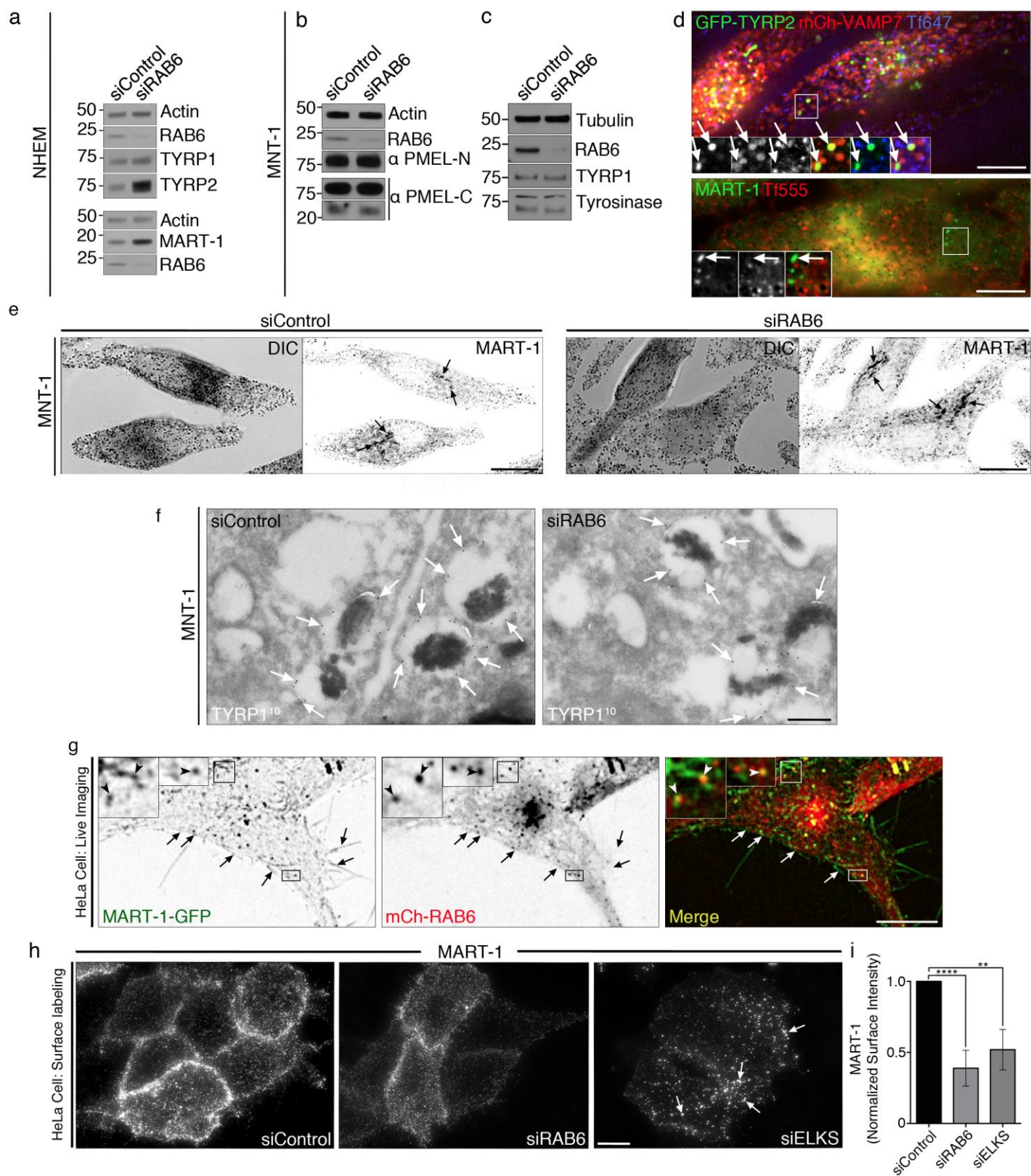
Supplementary Figure 4



Supplementary Figure 4: ELKS associates with activated endogenous RAB6-positive vesicles and to structures close to plasma membrane.

(a) WB of MNT-1 cell lysates treated with indicated siRNAs and probed for RAB6, ELKS, VAMP7 and Actin. (b) IFM on control- or ELKS-depleted MNT-1 cells imaged close to the glass (basolateral imaging plane Z-1) showed ELKS-positive structures close to plasma membrane (arrowheads, 5X area). (c) IFM on GFP-ELKS expressing MNT-1 cells stained for endogenous activated RAB6 (AA2) showed co-distribution on peripheral structures (arrows, 5X area). Scale bar: 20 μ m (b, c); M.W. in kDa.

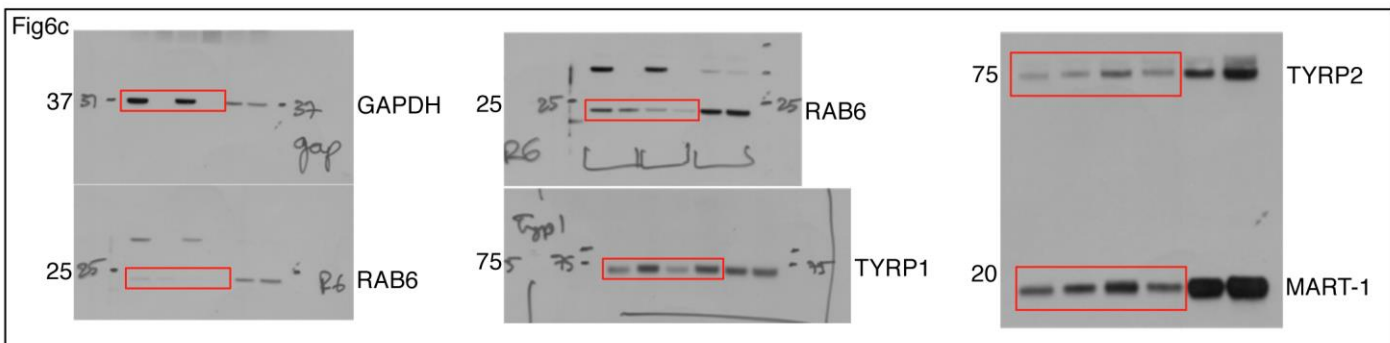
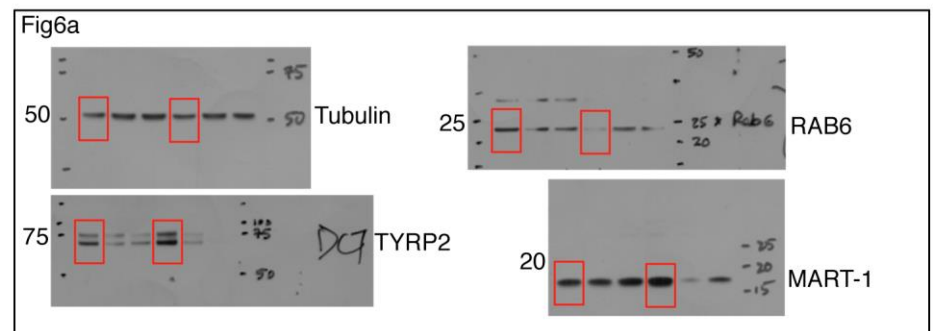
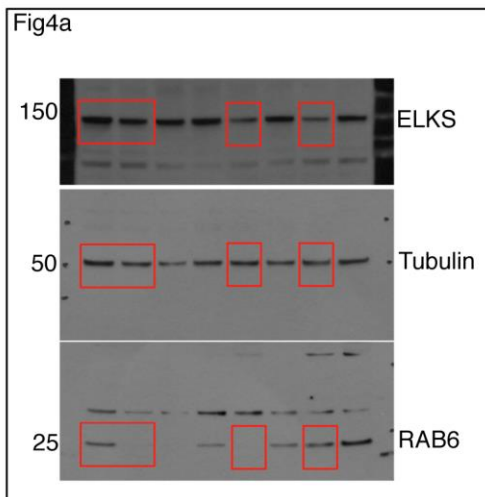
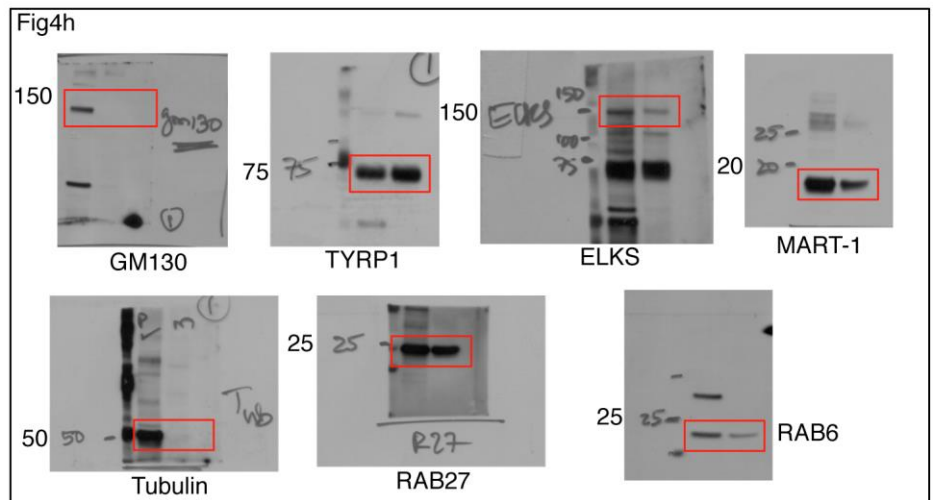
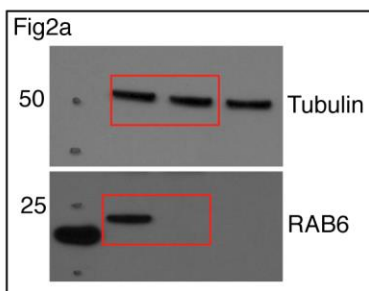
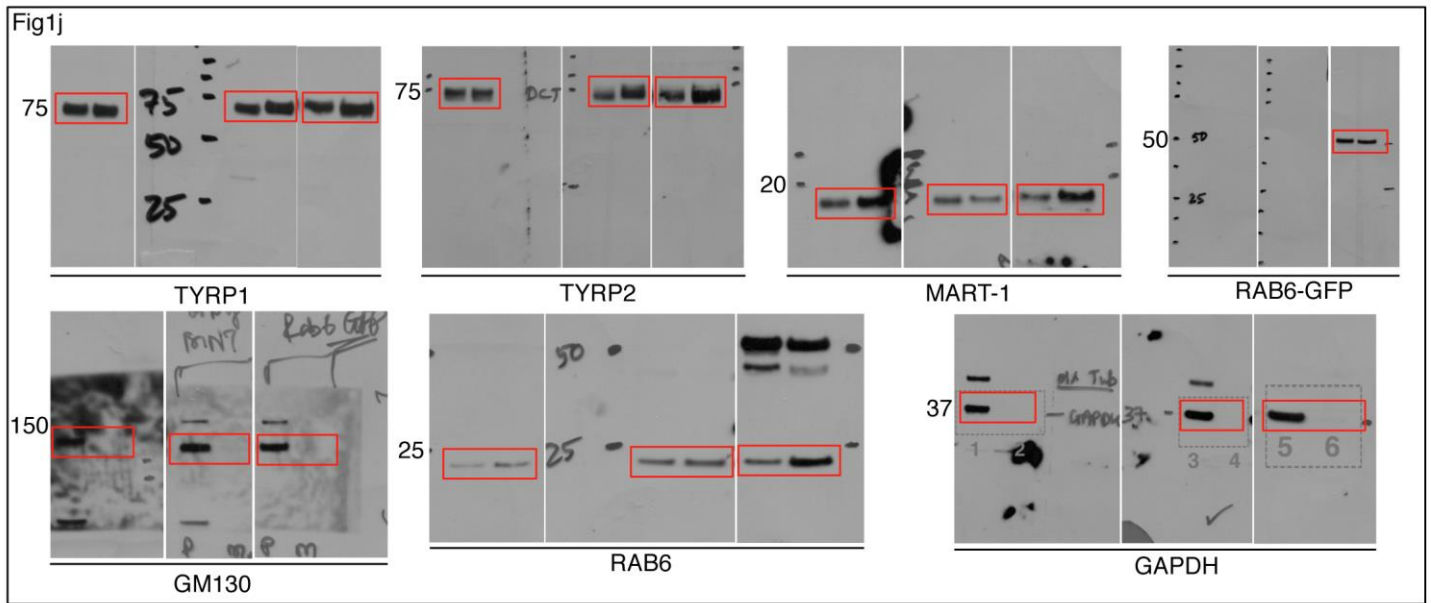
Supplementary Figure 5



Supplementary Figure 5: MART-1 and TYRP2 are specific cargoes of the RAB6/ELKS-dependent route.

(a) WB of control- or RAB6-depleted NHEM lysates probed for respective antibodies. (b-c) WB of control- or RAB6-depleted MNT-1 cell lysates showing no alterations in PMEL processing (b) and/ or TYRP1, Tyrosinase expression levels (c). (d, top) Live imaging frame of GFP-TYRP2 and mCh-VAMP7 co-expressing Tf-A647-internalized MNT-1 cells showing the co-distribution of TYRP2 with melanosomes (arrows, 2X area), but not with Tf-positive endosomes. (Bottom) IFM on TfA555-internalized MNT-1 cells stained for endogenous MART-1 did not show co-localization (arrows, 2X area). (e) IFM on control-or RAB6-depleted MNT-1 cells stained for endogenous MART-1 that accumulated at the Golgi area (arrows) upon RAB6 depletion, quantified in Figure 6h. (f) Immuno-labeling of ultrathin cryosection of control- or RAB6-depleted MNT-1 cells using TYRP1 antibodies (PAG-10 nm). TYRP1 localized to the limiting membrane of melanosomes (arrows). (g) Live imaging frame of HeLa cells co-expressing MART-1-GFP and mCh-RAB6. MART-1-GFP associated to the cell edges (arrows) and co-distributed with RAB6 on peripheral vesicles (arrowheads, 2X area). (h) MART-1 surface staining (using MART-1 N-terminal extracellular domain specific antibody) on non-permeabilised control-, RAB6- or ELKS-depleted HeLa cells expressing MART-1 untagged construct. RAB6 depletion decreased the overall staining, while the distribution appeared scattered (arrows) in ELKS depleted cells. (i) Quantification of cell surface staining of MART-1 in cells treated as in (h). Data are normalized to control and presented as mean \pm SEM (n=55-78 cells, 2 independent experiments). Scale bars: 10 μ m (d, e, g, h), (f) 200 nm; Two tailed, Unpaired t test (i); **, $P < 0.01$; ****, $P < 0.0001$; M.W. in kDa.

Supplementary Figure 6: Uncropped scans of the most important blots.



Supplementary Table 1: Analysis of Rab6 vesicles dynamics (mean \pm SEM)

siRNA	Cell	Vesicle	Track Time (sec)	Track time in Pause (%)	Average Velocity ($\mu\text{m}/\text{sec}$)	Pausing Vesicle (vesicle)	Pausing vesicle at melanosome (% of total pause)
-	12	80	15 ± 0.70	31 ± 2.6	0.88 ± 0.04	84% (69)	-
Control	16	112	15 ± 0.58	34 ± 2.2	0.84 ± 0.04	87.5% (98)	70.8%
ELKS	16	88	16 ± 0.74	18 ± 2.0	0.86 ± 0.05	63% (56)	37.7%